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Prevalence of transcription promoters within archaeal operons and coding sequences

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Despite the knowledge of complex prokaryotic-transcription mechanisms, generalized rules, such as the simplified organization of genes into operons with well-defined promoters and terminators, have had a significant role in systems analysis of regulatory logic in both bacteria and archaea. Here, we have investigated the prevalence of alternate regulatory mechanisms through genome-wide characterization of transcript structures of ~64% of all genes, including putative non-coding RNAs in *Halobacterium salinarum* NRC-1. Our integrative analysis of transcriptome dynamics and protein–DNA interaction data sets showed widespread environment-dependent modulation of operon architectures, transcription initiation and termination inside coding sequences, and extensive overlap in 3' ends of transcripts for many convergently transcribed genes. A significant fraction of these alternate transcriptional events correlate to binding locations of 11 transcription factors and regulators (TFs) inside operons and annotated genes—events usually considered spurious or non-functional. Using experimental validation, we illustrate the prevalence of overlapping genomic signals in archaeal transcription, casting doubt on the general perception of rigid boundaries between coding sequences and regulatory elements.

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Subject Categories: functional genomics; chromatin & transcription

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Introduction

Systems-biology approaches have been successfully applied to construct quantitative and predictive models of biological networks (Bonneau *et al*, 2007; Faith *et al*, 2007). However, a significant amount of information is missing from these models because of incomplete parts lists (unannotated genes, non-coding RNAs (ncRNAs), poorly understood protein modifications and so on) as well as a lack of molecular detail associated with these processes. Incorporating such detail will make these models mechanistically accurate and useful for synthetic-biology approaches targeting large-scale biological-circuit re-engineering. Among the current systems-scale models most amenable for such large-scale redesign are those that describe gene-regulatory networks (GRNs).

GRN models are usually built upon transcriptome data, in which typically genes or gene modules (with similar expression patterns and shared regulatory motifs) are associated with their transcriptional regulators through linear or Bayesian models. However, although these models can be predictive (Bonneau *et al*, 2007), they often rely on approximations of the transcription process and lack finer details of dynamic environment-dependent assembly of transcription complexes at each of the numerous promoters in the genome. High-density tiling arrays can be used to define transcribed regions (David *et al*, 2006), start sites (McGrath *et al*, 2007), and protein–DNA interaction sites (Reiss *et al*, 2008), which can be used to identify some of these missing details associated with transcriptional regulation, and thereby enable us to construct systems-scale predictive models of GRNs that are also mechanistically accurate.

We recently constructed a model of an environment and gene-regulatory influence network (EGRIN) for the halophilic archaeon *Halobacterium salinarum* NRC-1. This model accurately predicts the transcriptional changes in 80% of all genes to new environmental and genetic perturbations (Bonneau *et al*, 2007). Using an integrated biclustering algorithm to identify regulons and their putative *cis*-regulatory motifs (Reiss *et al*, 2006), and a sparse regression procedure to statistically pair these regulons with their putative regulators (Bonneau *et al*, 2006), we were able to discover the combinatorial and conditional regulation of genes by multiple TFs and EFs (environmental factors) (Bonneau *et al*, 2007). Although several of the statistically inferred influences in this network were shown to be likely mediated through direct interactions with the promoters of regulated genes, a large number of influences are thought to be indirect. The logical next step is to make this quantitative and predictive network also mechanistically accurate on a systems scale.

Construction of a mechanistically accurate systems-scale model is a reasonable expectation for *Halobacterium salinarum* NRC-1, as its transcription is driven by a simplified version of a eukaryotic RNA polymerase (RNAP) II (Hirata *et al*, 2008) in a genome with prokaryotic organization. The archaeal RNAP requires only two general transcription factors – GTFs (TATA binding protein –TBP and transcription factor B –TFB) for promoter recruitment and basal transcription initiation. Furthermore, only ~130 putative transcriptional regulators (TRs) are present among the ~2400 genes encoded in the genome of *H. salinarum* NRC-1 (Ng *et al*, 2000). A relatively small number of genes and few TFs (GTFs and TRs) together make *H. salinarum* NRC-1 an attractive model system for characterizing gene-regulatory mechanisms at all promoters. Notably, the combinatorial action of multiple TFs and TBPs (*H. salinarum* NRC-1 possesses 6 TBPs and 7 TFs) in defining basal promoter architecture in most archaea (Baliga *et al*, 2000; Facciotti *et al*, 2007) provides a unique opportunity to characterize dynamic conditional regulation of a large fraction of genes during cellular responses to complex changes.

Here, we report a significant step toward a mechanistically accurate EGRIN model by characterizing the dynamic remodeling of the transcriptome structure of *H. salinarum* NRC-1 during a complex cellular response, and correlating these changes to genome-wide binding locations of 50% of all predicted GTFs as well as several specific TRs. By integrating diverse data types, we identified: (i) transcription start sites (TSSs) and termination sites (TTSs) for ~64% of the genes, including new and revised protein-coding genes; (ii) 61 new

ncRNA candidates; (iii) 5' and 3' untranslated regions (UTRs) of mRNAs; (iv) functional promoters upstream and internal to coding regions; (v) instances of transcription termination inside coding sequences; (vi) mRNA populations with variable 3'-end locations; (vii) transcripts with extensive overlaps in their 3' termini; and (viii) operon-encoding transcripts of variable length. Significantly, these findings suggest that the incorporation of mechanistic accuracy into GRN models would require genes, operons, promoters, and terminators to be treated as dynamic entities.

Results

Genome-wide protein–DNA binding data show TF binding inside genes and operons

A detailed map of genomic locations where TFs bind DNA and modulate transcription is essential to model mechanisms of gene regulation on a systems scale. Chromatin immunoprecipitation of transcription complexes coupled to microarray (ChIP–chip Ren *et al* (2000)) or sequencing (ChIP–seq (Robertson *et al* (2007))) is a commonly used approach to construct such maps. In ChIP–chip, the resolution to which the protein–DNA binding sites (TFBSs) can be identified is often limited by the genomic spacing of the probes in the array. We utilized the *MeDiChI* algorithm (Reiss *et al*, 2008) to estimate precise TFBS locations and their corresponding local false discovery rates (LFDRs) from new and previously reported genome-wide ChIP–chip measurements for 11 TFs (with two or more biological replicates for each): all TFs (TFBa, TFBb, TFBc, TFBd, TFBf, TFBg, one TBP (TBPb) and three TRs (Trh3, Trh4, and VNG1451C) in *H. salinarum* NRC-1 (see Materials and methods). On the basis of simulations similar to those of Reiss *et al* (2008), with a noise model customized to mimic the data used in this study, we estimated that the average positional uncertainty in TFBS locations identified by *MeDiChI* averaged ~50 nucleotides (nt) (1SE) over all ChIP–chip data sets used in this study.

We found that the 3072 significant (LFDR < 0.1) individual TFBSs for all data sets often fell within distinct loci where at least three different TFs were observed within a ± 50 nt window ($P < 10^{-8}$). We therefore refined this TFBS list to a conservative set of 318 such distinct ‘multi-TF-binding loci’, hereafter TFBS loci throughout the genome (Table 1; see Supplementary Table 1 for each loci). As we applied to each individual data set an LFDR cutoff of 0.1, which by itself is rather stringent, the joint LFDR of these 318 TFBS loci is

Table 1 Numbers of TFBS loci comprised of varying numbers of individual TFBS and their distribution in annotated coding sequences, predicted operons, and conditional predicted operons

Number of loci	Total	In annotated coding sequence	In predicted operons	In conditional predicted operons (P-value)
With ≥ 1 TFBS	1249	368	82	58 (1.4×10^{-10})
With ≥ 2 TFBS	649	231	34	28 (4.3×10^{-8})
With ≥ 3 TFBS	318	96	13	13 ($< 1 \times 10^{-30}$)
With > 3 TFBS	196	56	10	10 ($< 1 \times 10^{-30}$)

The reported results of this paper utilize the 318 very stringent ≥ 3 TFBS loci but clearly the same conclusion holds (although the numbers increase) as this threshold is relaxed. The P-values were estimated for the probability of observing as many TFBS loci internal to conditional operons (column 4), given the number of TFBS loci observed internal to all operons (column 5), and the estimated fraction of conditional operons (~43%; see Results and Discussion).

significantly smaller than that. Although each individual TF had a significant bias of binding in annotated intergenic regions ($\sim 60\%$, on average, versus $\sim 16\%$ expected), this fraction increased to $\sim 70\%$ (276) when considering the 318 TFBS loci ($P \sim 10^{-31}$). Monte Carlo simulations of TFBSs placed only in non-coding regions in the genome with a ~ 50 – 75 nt positional uncertainty and an LFDR between 0.1 – 0.01 show that 80 – 85% of detected TFBSs should fall in intergenic regions (for more details, see Materials and methods). Thus, our assessment was that a small but significant fraction of these significant TFBS loci in our ChIP–chip data sets (as many as $\sim 10\%$ of the multi-TFBS loci) fell within coding regions. Here onwards, we present detailed and systematic experimental validation that shows that many of these TF-binding events inside coding sequences have significant consequences on the transcriptional regulation of diverse aspects of cellular physiology.

Analysis of transcriptome structure shows new expression features

The location of a TFBS in the vicinity of a TSS or a TTS could indicate whether a given binding event is functional, especially for the interactions localized within a gene or operon. We investigated this by systematically mapping transcript boundaries and their dynamic changes at the whole-genome level using genome-wide tiling array data and then integrating this information with the TF-binding information.

We define transcriptome structure as the collection of TSSs and TTSs that together characterize transcriptional units (mono- and polycistronic mRNAs, tRNAs, rRNAs, and other ncRNAs). Sequence signatures for these features are yet to be characterized in archaea, and computational predictions based on known signatures in bacteria and eukaryotes remain error prone due to incomplete understanding of transcription processes in all organisms (Jones, 2006). Therefore, we experimentally mapped the transcriptome structure of

H. salinarum NRC-1 by hybridizing total RNA (including RNA species <200 nt) to genome-wide high-density tiling arrays (60mer probes with 40 nt overlap between contiguous probes).

We first applied a segmentation algorithm based on regression trees (see Materials and methods) to map transcript boundaries in cells cultured under standard laboratory growth conditions (mid-logarithmic phase, 37°C , 225 r.p.m. shaking—hereafter ‘reference RNA’) (Figure 1A). Although this approach effectively mapped TSSs for mRNAs, tRNAs, rRNAs and probable ncRNAs with significant expression levels, it was ambiguous for genes with low expression levels. Moreover, TTSs proved difficult to determine in general, even for highly expressed genes, because no sharp boundaries were observed for most transcripts at the 3′ termini (Figure 1A; Supplementary Figure 1B). We overcame these challenges and recovered further information by analyzing dynamic modulation of the transcriptome structure during typical growth of a batch culture under standard conditions (Figure 1B).

H. salinarum NRC-1 presents a number of interesting switches in metabolism during growth (Facciotti *et al*, submitted) because of complex changes in EFs, including pH, oxygen, nutrition, and so on (Schmid *et al*, 2007). Although most single perturbations (radiation, oxygen, metals, and so on) affect the expression of only $\sim 10\%$ of all genes (Baliga *et al*, 2004; Kaur *et al*, 2006; Whitehead *et al*, 2006), the changes during growth resulted in differential regulation of a significantly higher proportion of genes ($\sim 63\%$, 1518 genes) (Figure 1B). These conditions thus enabled the investigation of a wider transcriptional landscape, which includes not only modulation of transcript levels (Figure 1B), but also extensive changes in transcriptome structure. We observed altered TSSs, TTSs, operon organizations, and differential regulation of putative ncRNAs (Supplementary Figure 1). By integrating hybridization signals (Figures 1A and 2B) with dynamic growth-related changes (Figure 2C and D), we estimated the probability that each tiling

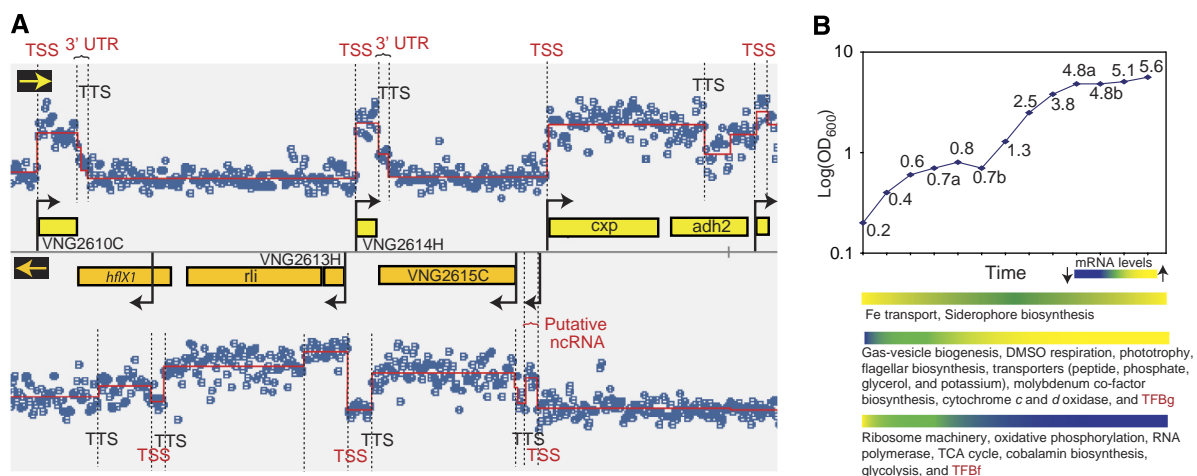
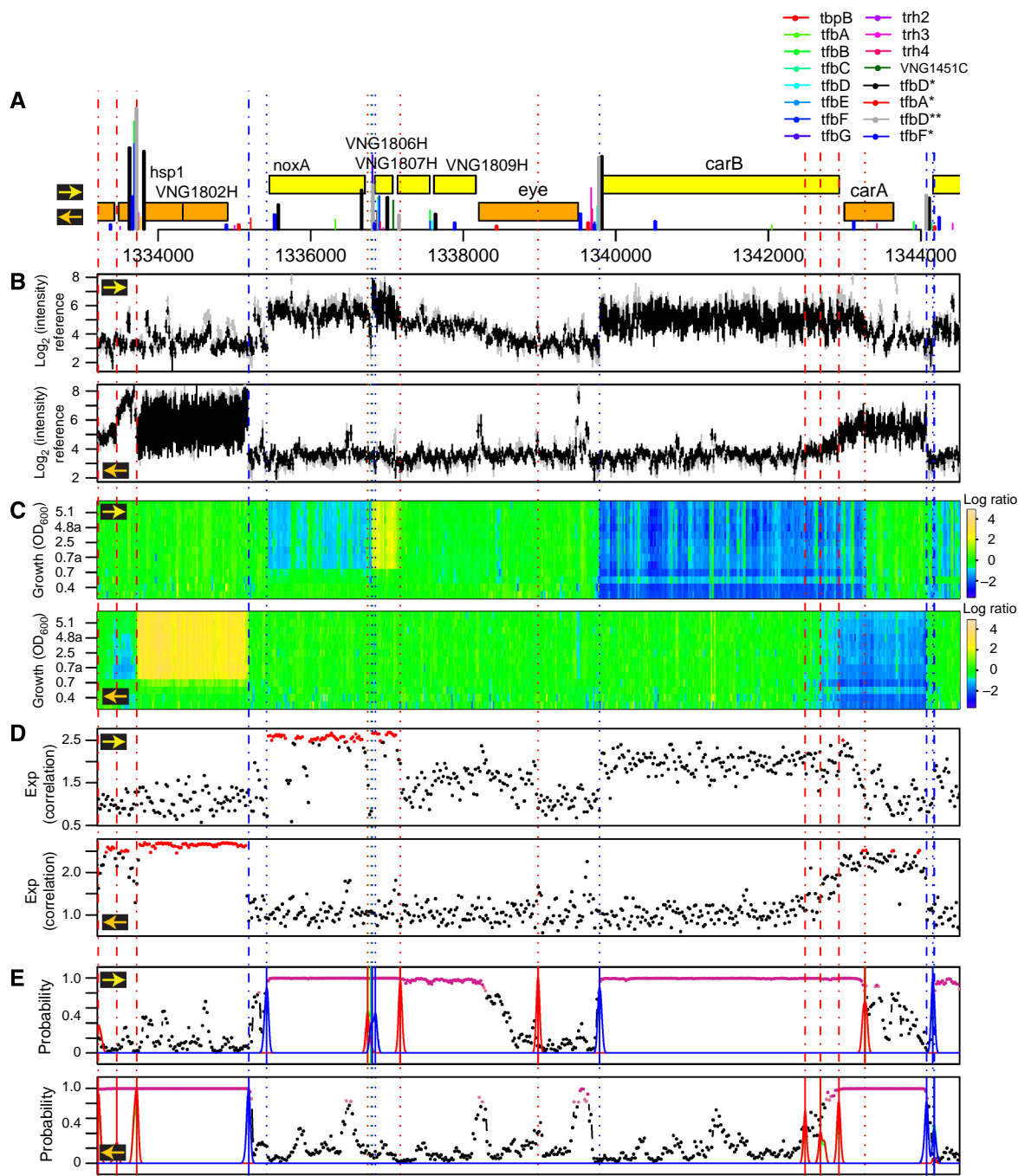


Figure 1 Transcriptome structure and growth-phase-dependent changes in *Halobacterium salinarum* NRC-1. **(A)** Genome map of a segment of the main chromosome of *H. salinarum* NRC-1 (NC_002607) with corresponding signal intensity of total RNA from a mid-log phase culture (‘reference RNA’) hybridized to 60mer overlapping probes in a high-density tiling array. Genes in the forward and reverse strands are shown in yellow and orange, respectively. Each blue dot represents probe intensity (in \log_2 scale) at the given genomic location in the forward (upper panel) or reverse (lower panel) strands. The overlaid red line is the result of a segmentation algorithm that was applied to determine transcription start sites (TSS and black arrows), transcription termination sites (TTS), untranslated regions in mRNAs (3′ UTR), and putative non-coding RNAs. **(B)** Dynamic changes in transcriptome structure were evaluated (Figure 2) at different phases of growth in a standard laboratory batch culture. Important physiological changes that are reflected in differential expression of corresponding mRNAs during the various phases of growth are indicated with a heat map (Facciotti *et al*, submitted).



array probe was complementary to a transcribed region, mapped locations of putative transcript boundaries (Figure 2E; see Materials and methods) and identified 1574 TSSs and 1952 TTSs for most genes with some transcriptional variation. Subsequently, we manually assessed and curated gene assignment to each TSS and TTS. The error of these assignments is given by the resolution of probes on the tiling array (20 nt). In sum, TSSs were assigned to 64% (1156 singletons and 544 genes in 203 operons) of all annotated genes and TTSs were assigned to 1114 genes and 202 operons. A TSS and a TTS together define a unit of transcription (Supplementary Table 2). We describe below, how by correlating locations of these transcriptional units to predicted coding sequences in the genome, we were able to characterize and discover new features within the transcriptome structure.

- a. *Transcription of mono- and polycistronic mRNAs.* In many organisms, especially prokaryotes, genes of related function are often co-transcribed as a single polycistronic mRNA (operons). Operon predictions based on genome-specific distance models, combined with comparative genomics and functional features identified 299 operons in *H. salinarum* NRC-1 (Price *et al*, 2005). According to our analysis of 1,698 genes with significant transcription signal, at least 544 (32%) genes were transcribed as polycistronic mRNAs in 203 operons. Comparative analysis with the predicted operon structures identified 123 new or truncated operons, which are dynamically regulated during growth.
- b. *Discovery of leaderless transcripts and 5' and 3' UTRs.* UTRs in the proximal (5') end of transcripts often contain signals, such as the Shine–Dalgarno (SD) sequence signature for ribosome loading (Sartorius-Neef and Pfeifer, 2004). Although some mRNAs spanned short distance beyond the coding-sequence boundaries, others were significantly longer (greater than the error in transcript-boundary assignment–20 nt), with 5' (457 transcripts, 40% of genes assigned to an experimentally determined TSS) and/or 3' (857 transcripts, 77% of the genes assigned to an experimentally determined TTS) UTRs (Supplementary Table 2 and Supplementary Figure 2). We validated the TSS and 5' UTR lengths by comparing our observed UTR lengths with those experimentally measured in a closely related strain *-H. salinarum* R-1 (Brenneis *et al*, 2007). We found that, on average, the predicted 5'-UTR lengths correlate strongly with those determined by Brenneis *et al* (2007) ($P < 0.001$); however the predicted NRC-1 3' UTRs are usually longer (on average 1.8 ± 1.3 times longer than those of R-1) (Supplementary Figure 2). Interestingly, 137 transcript pairs had overlapping 3' ends (Supplementary Table 3) ranging from 25 to 788 nt in length, with a median length of 264 nt.
- c. *Distance between newly mapped TSSs and GTF-binding sites agrees with earlier knowledge of GTF binding.* It is known that the archaeal pre-initiation complex lies between 25–30 nt upstream of the TSS (Bell *et al*, 1999). Although the relatively large uncertainty in the MeDiChI-mapped TFBSs precludes the quantification of this distance for individual TSSs, we found that the 318 TFBS loci (defined above) lie at an average of 24 nt (95% probability that the average falls between 35 and 16 nt) upstream of the nearest TSS. This may be compared with an average upstream

distance of 59 nt (95% probability that the average lies between 69 and 49 nt) between the TFBS loci and the first (annotated) translation codon. This difference is further evidence of the significant number of genes with 5' UTRs (see above).

- d. *Revisions of predicted translation start sites and discovery of new protein-coding genes.* For 222 transcripts (178 genes and 31 operons), we observed that the TSS is downstream (by > 20 nt) of the predicted start codon (Ng *et al*, 2000) and that the TSSs for 45% of these (52% of the genes and 26% of the operons) are corroborated by the locations of one or more TFBSs. We investigated this further by analyzing the distribution of peptides detected in 527 tandem mass spectrometry runs from 91 proteomics experiments representing 121 618 high-quality tandem mass spectra within the *H. salinarum* NRC-1 peptide atlas (PA) (Van *et al*, 2008). This study added 30 runs in four new experiments conducted specifically to catalog the proteome under conditions in which the reference RNA was prepared. Absence of peptides from these apparently untranscribed regions led to the revision of start codons for 61 genes and 12 operons. Conversely, we were also able to match previously unassigned tandem mass spectra to 10 new transcripts representing new protein-coding genes, and to detect longer proteins and transcripts for three genes (Supplementary Table 4). Comparison with strain *H. salinarum* R-1 (Pfeiffer *et al*, 2008) showed that most of these discrepancies with the original genome annotation of *H. salinarum* NRC-1 had been resolved in the newer annotation.
- e. *Discovery of putative ncRNAs.* Non-coding RNAs are implicated in diverse regulatory processes from chromatin accessibility to mRNA translation and even modulation of protein activities (Storz *et al*, 2005). In archaea, 57 ncRNAs have been identified in *Sulfolobus solfataricus* (Tang *et al*, 2005) and 86 in *Archaeoglobus fulgidus* (Tang *et al*, 2002) by cDNA library cloning. Little is known about their functions except for the well-characterized snRNAs (Dennis and Omer, 2005). Although reliable bioinformatics approaches exist for identifying tRNAs and rRNAs, detection of other ncRNAs remains a challenge (Wang *et al*, 2006). We identified at least 61 transcripts experimentally with no bona fide coding sequence and/or matching tandem mass spectra from diverse proteomic analyses (Supplementary Table 5). These sequences were defined as putative ncRNAs. Fifty-two ncRNAs (85%) had increased expression levels at higher cell densities, whereas only 9 (15%) were down-regulated. This is consistent with the predicted function of ncRNAs during stress (Shimoni *et al*, 2007), given the decrease in oxygen and nutrient availability at higher cell densities. Expression profiles for most ncRNAs were either positively or negatively correlated with corresponding changes in antisense transcripts ($P < 10^{-14}$) (Figure 3B).

Integration of TFBSs with transcriptome structure shows conditional modulation of operon organization

Changes in transcript levels and structure are ultimately the product of gene regulation mediated by dynamic assemblies of

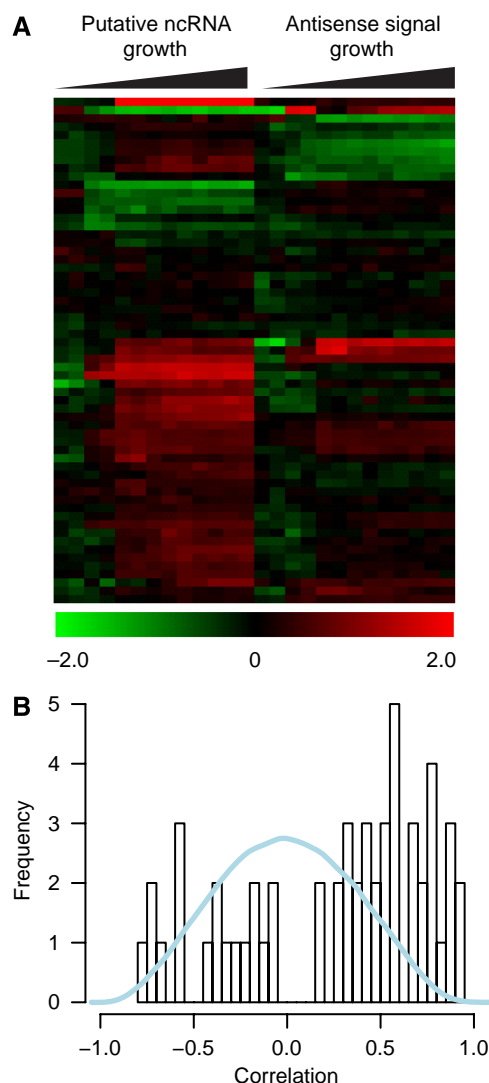


Figure 3 New non-coding RNAs in *Halobacterium salinarum* NRC-1. (A) Expression profiles of 61 putative ncRNAs and their respective antisense transcripts during growth. (B) The bimodal distribution of correlations between putative ncRNAs profiles and antisense transcripts suggests the ncRNAs might stabilize or destabilize transcripts on the opposite strand (the null distribution from randomly selected probes is shown in blue).

TF-DNA complexes. This mechanistic perspective of global gene-expression dynamics can be obtained by correlating transcript boundaries with genome-wide binding locations of TFs. We estimated that at least 13 of the 318 TFBS loci were internal to predicted operons (Price *et al*, 2005) (see Materials and methods). This is a conservative estimate (see Table I) as only TFBS with three or more binding loci were considered. Eight of these TFBS loci fall within 50 nt of an intergenic ‘gap’ between predicted open-reading frames in the operon. An interesting example is the cluster of genes involved in arginine fermentation (*arcRACB*), at least two of which (*arcC* and *arcB*) constitute a predicted operon (Figure 4A) (Price *et al*, 2005). Notably, the strong co-expression of these genes across a wide range of environments (Bonneau *et al*, 2007) (Figure 4A(a))

does not necessarily result from a unique polycistronic message. The GTF-binding sites upstream to *arcC* and in the intergenic region between *arcC* and *arcB* (Figure 4A(b)) along with nearby TSSs (Figure 4A(d)) indicate two promoters that yield three possible transcripts: polycistronic *arcCB* and monocistronic messages for each *arcC* and *arcB*. The three possible transcripts suggested by our analysis (polycistron *arcCB* and monocistronic messages for each *arcC* and *arcB*) were consistent with previously reported northern-blot experiments that identified all three transcripts (Ruepp and Soppa, 1996), showing that our systems-level approach was able to independently recover known information.

This prompted us to search for operons interspersed with conditionally active promoters. For instance, we observed a TFBS internal to the operon *VNG2211H-endA-trpS1* (Figure 4B). This putative operon encodes a hypothetical protein, a tryptophanyl tRNA synthetase and a tRNA intron nuclease, respectively (Figure 4B(b)). The internal TFBS correlates with both a nearby TSS and differential expression of the operon genes during growth: whereas *trpS1* is down-regulated, expression levels of *endA* and *VNG2211H* do not change (Figure 4B(c)). Although these genes are co-expressed in most of the ~700 microarrays representing ~20 different environmental perturbations, we could detect instances besides standard growth where they are not co-transcribed, such as during interaction of *H. salinarum* NRC-1 with *Dunaliella salina*, a unicellular alga (Figure 4B(a), green box). Notably, the conditionally activated promoter for *trpS1* is located within the coding sequence of *endA*.

Using this approach, we manually identified 78 operons with conditionally altered gene-expression levels of constituent genes. To gain a global perspective on the prevalence of conditional operons, we classified all predicted operons in the *H. salinarum* NRC-1 genome based on two scores (Figure 4F): (1) correlations between expression profiles of genes in each operon among 719 microarrays and (2) the ‘tiling score’, which is the $\log_{10}(P\text{-value})$ of the statistical significance of the difference in tiling array probe intensities or ratios between the genes in the operon (Materials and methods). A low ‘tiling score’ indicates operons where member genes present significantly different transcript levels in the reference RNA or in any of the growth samples probed. We then classified the operons using the manually identified conditional operons as the training set (Figure 4F, green circles). Even for operons with genes that are co-expressed in most environmental conditions (high correlation), such as the *arcC-arcB* operon (Figure 4A(a)), the low tiling score (Figure 4F and 4A(d)) accurately identifies an internally located alternate promoter. The operon *VNG2211H-endA-trpS1* has a low correlation score, as these genes are anti-correlated in some conditions (Figure 4B(a)), as well as a low tiling score because of significant difference in their absolute expression levels (Figure 4B(c)). Other examples of conditional operons are illustrated in Figure 4: *sdhCDBA* encodes the subunits of the succinate-dehydrogenase complex (Figure 4C), where *sdhC*, *D*, and *B* are downregulated at high cell densities, whereas the expression of *sdhA* remain unchanged (Figure 4C (c)); *dppFDB2C2*, which encodes the subunits of a dipeptide ABC transporter (Figure 4D), in which, with the exception of *dppF*, all other genes are upregulated at higher cell densities

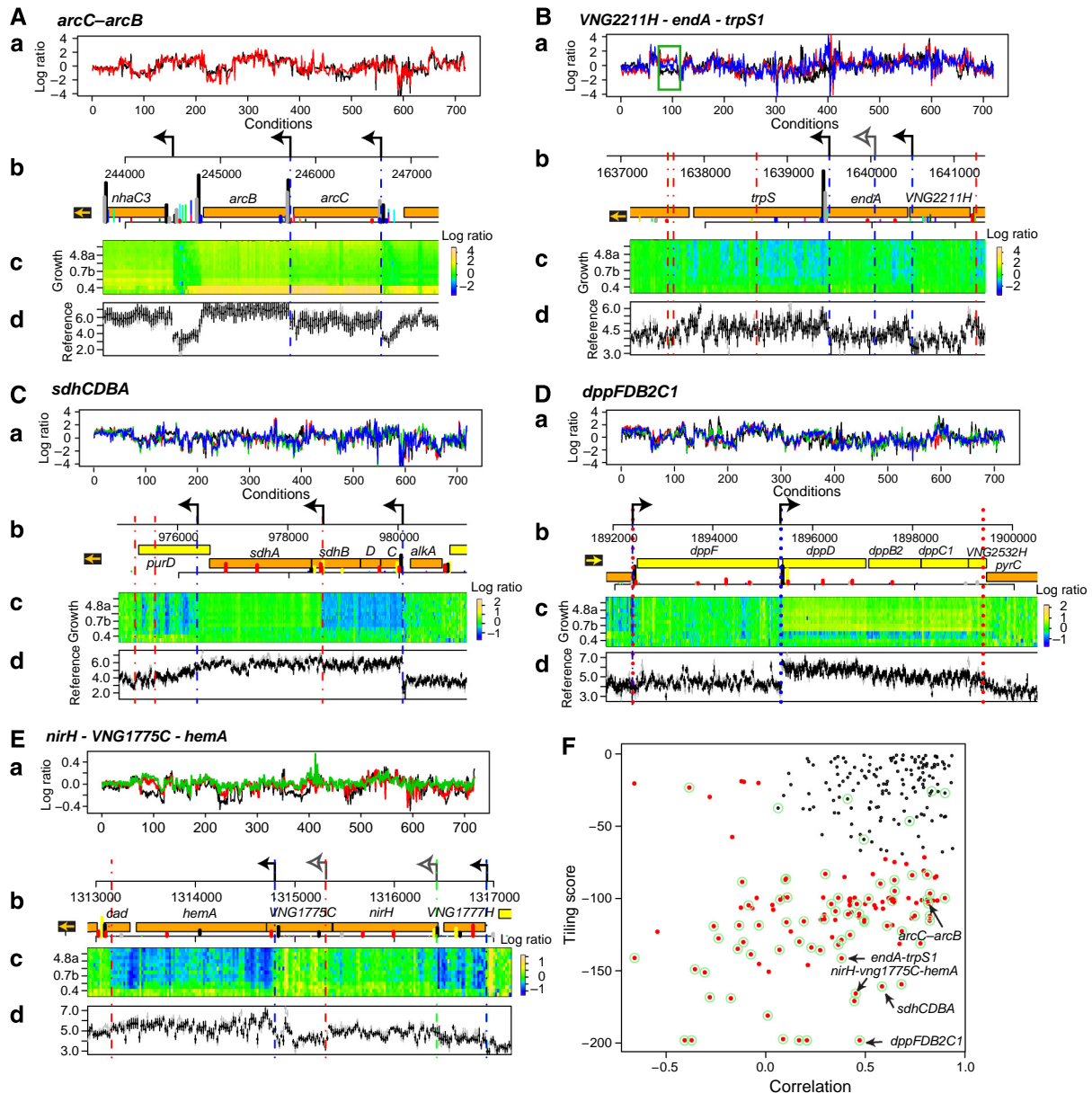


Figure 4 Conditional modulation of operon organization. Analysis of predicted operon structures identifies unexpected internal promoters that conditionally break the organization during cellular responses in differing environments. (**A**) The high degree of co-expression of *arcC* (red) and *arcB* (black) transcript-level changes in diverse environments (probed by ~700 microarray experiments) (a) coupled to their genomic organization (b) strongly suggested co-transcription of these genes as an operon. Dynamic transcriptional changes of these genes during growth (c) also support this prediction. However, the integrated transcriptome-structure analysis identified a promoter (black arrow along genome coordinates of plasmid pNRC200 (NC_002608) in (b) and vertical blue line spanning panels b–d) in the 56-nt intergenic region between *arcB* and *arcC*. The location of the promoter is consistent with the different absolute levels of transcripts spanning the two genes (d) as well as with locations of TFBSs (vertical lines in the pNRC200 map in (b); for color code see Figure 2). (**B**) Although the predicted operon organization of *VNG2211H* (blue), *endA* (red), and *trpS1* (black) is supported by their co-expression in most environments, their expression is not correlated during a few responses, including experiments investigating *H. salinarum* NRC-1 interaction with a unicellular alga (green box) (a). This differential regulation was also observed during growth (c) and could be explained by an alternate promoter within the coding sequence of *endA* (black arrow) whose location was corroborated by co-localized TFBSs (b) and a distinct TSS (c and d). A second weak TSS was also identified internal to *endA* (gray open arrow). (**C**) Genes in the predicted operon *sdhCDBA* (*sdhC* - blue, *sdhD* - green, *sdhB* - red, and *sdhA* - black) are co-expressed in most of the environmental perturbations, except for *sdhA* during a few responses. (b) TFBS (vertical lines, color coded as Figure 2) are found near the TSS for *sdhC* and in the coding region of *sdhB* (black arrows, blue dashed lines). (c) Dynamic changes during growth show that *sdhCDB* is downregulated and *sdhA* does not have the expression levels altered (d) and reference-RNA hybridization shows that *sdhA* is expressed. (**D**) Operon *dppFDB2C1*. (a) *dppF* (black) and *dppD* (red), *dppB2* (green) and *dppC1* (blue) are organized in a predicted operon and are co-expressed in most of the environmental perturbations. TSS identified for *dppF* and *dppDB2C2* (black arrows and blue dotted lines) are localized near (b) TFBS (vertical lines, color coded according to Figure 2), which could explain the (c) differential expression of *dppF* and *dppDB2C1* during growth. (**E**) Operon *nirH-VNG1775C-hemA*. (a) *nirH* (green), *VNG1775C* (red), and *hemA* (black) are organized in a predicted operon and co-expressed in most of the environmental perturbations. (b) TFBS localized internal to *VNG1775C* (vertical lines) are found near the TSS for *hemA* (black arrow), which could explain (c) the differential expression of this gene at higher cell densities. (**F**) Conditional operons were identified in a genome-wide manner by analyzing two parameters: minimum correlation score along all 719 environmental conditions between each gene in each predicted operon (horizontal axis) and minimum 'tiling score', which quantifies the difference in the tiling probe levels for genes constituting the operon (vertical axis; see Results and Discussion for details). Green circles represent operons that were manually identified as condition dependent and were used as a training set for the conditional-operon classification. Red dots represent operons that were automatically classified as condition dependent (see Materials and methods for details). The conditional operons described above are highlighted.

(Figure 4D(c)) and *nirH-VNG1775C-hemA* (Figure 4E), where *hemA*, encoding a glutamyl-tRNA reductase is downregulated at high cell densities.

We were able to compute tiling and correlation scores for the 269 operons in *H. salinarum* *NRC-1* with significant expression in the tiling-array experiments, and classified 115 (~43%) as condition dependent (Figure 4F, red dots; Supplementary Table 6). Interestingly, conditional operons were highly enriched for internal TFBS loci relative to their non-conditional-classified peers ($P < 10^{-9}$, Figure 4F, black dots); indeed all 13 operon-internal TFBS loci fell within conditional operons ($P < 10^{-30}$, see Table I).

Interaction of TFs within coding regions is associated with transcript boundaries

The operons in *H. salinarum* *NRC-1* usually have very short (~50 nt) or no intergenic regions between constituent annotated coding regions ('gaps'). We find that although only 27% of the gaps between coding regions in all 299 predicted operons are longer than 20 nt, this fraction increases to 37% in the conditional operons (Supplementary Figure 3). Although this might partly explain some of the internal promoter activity, the lack of a significant intergenic region (≤ 20 nt) between at least 53 conditionally co-transcribed gene pairs within operons suggest the presence of alternate internal promoters within coding sequences, as illustrated with operons *VNG2211H-endA-trpS1*, *sdhCDBA*, and *nirH-VNG1775C-hemA*. Notably, absence of a significant intergenic gap and high degree of correlation in transcription profiles for these genes would have precluded discovery of their condi-

tional co-transcription based on generally accepted rules for operon organization (Supplementary Figure 3E).

TF binding in the middle of coding sequences can also result in transcription initiation or termination internal to a single annotated protein-coding gene. We highlight this with an example that focuses on gas-vesicle biogenesis—a hallmark response of *H. salinarum* *NRC-1* to low toxic conditions under high cell density (Yang and DasSarma, 1990). Several TFs, including TFBd, bind internally to two distinct loci within *gvpE1*, a transcriptional regulator of gas-vesicle biogenesis (Scheuch *et al*, 2008). The binding locations of one set of TFs correlates with the termination of a transcript initiated upstream to *gvpD*. Moreover, when we observe the relative transcript levels in a strain overexpressing TFBd, it results in upregulation of a transcript downstream of its binding location in the second locus (Figure 5d). Together these results show both a regulated TFBd-dependent promoter and a growth-phase dependent terminator located inside the *gvpE* coding sequence. It is noteworthy that despite extensive prior analysis of the transcriptional regulation of gas-vesicle biogenesis, this aspect of growth-dependent regulation was never discovered.

We note that our estimate of the prevalence of internal TFBSs as described above is conservative given the stringent nature of our automated analysis with the inclusion of only significant multi-TFBS loci, as well as the limited range of conditions under which both transcriptome and ChIP-chip data were collected. Indeed, although 69% of the 318 TFBS loci lie in intergenic regions, a fraction of the remaining 31% (~100 sites) which fall within annotated coding regions (in particular, 42 of these, which fall > 50 nt from any annotated start or stop site) are likely functional (Table I). After revising the predicted translation start sites based on the transcriptome

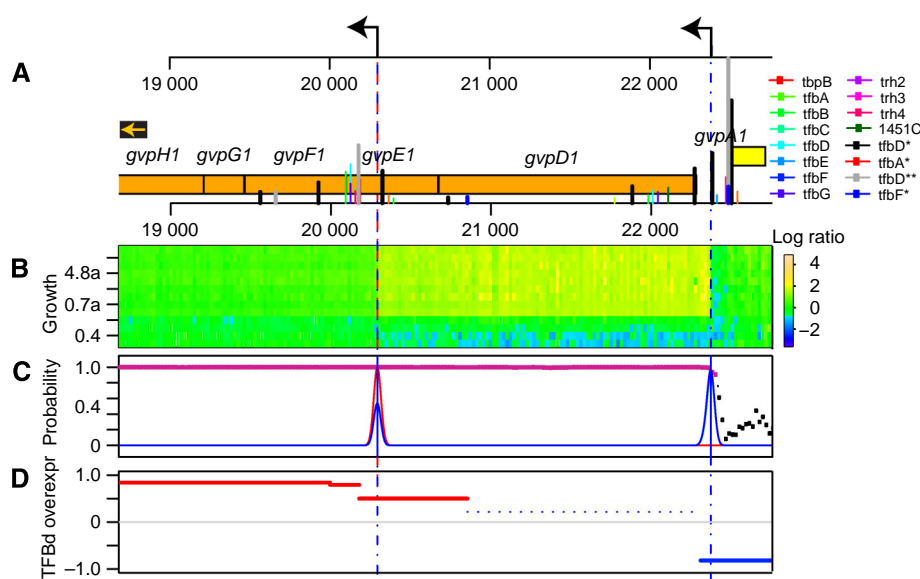


Figure 5 TF binding internal to coding regions results in transcriptome-structure changes. A putative promoter and a terminator internal to the coding sequence of *gvpE1*, a gas-vesicle biogenesis regulator, is corroborated by co-localized TFBSs for several TFs, including TFBd (A). Although the activity of the terminator was verified by growth-phase-dependent termination of transcription originating upstream to *gvpD1* (B), this region also presented high probability of being transcribed ($P > 0.9$ are highlighted in pink) and a putative transcription start site from an internal promoter (blue line) (C); the internal promoter could be validated by analyzing the transcriptome structure in a strain overexpressing TFBd (D). The red line indicates a break in the transcription levels of the strain overexpressing TFBd relative to the reference RNA. This evidence associated with mapped TFBd-binding site and TSS suggests the presence of an internal promoter.

structure as described previously, we observed 610 TFBSs over all 11 TFs (LFDR < 0.1) that fell within coding regions (by > 50 nt) and 47 (7.7%) were nearby (within 100 nt) a putative internal transcription break point ($P \sim 0.015$ relative to randomly placed internal break points), suggesting that they might constitute functional promoters and/or terminators (Supplementary Table 7).

However, using the automated procedure, about half of all detected TSSs for annotated genes were not associated with any detectable TFBSs and about half of all detected TFBSs were not associated with any identifiable TSS. Although a significant fraction of transcript boundaries might also result from alternate regulatory mechanisms, such as transcript cleavage, our inability to correlate these features to TF-binding locations might also reflect the dynamics and complexity of combinatorial TF binding and TSS selection.

Discovery of conditional promoter binding of GTFs

All ChIP-chip data described above were collected at mid to late phase of growth in batch cultures ($OD_{600} > 1.0$), and therefore are not specific to the conditions over the entire growth curve, which were investigated in the mRNA-expression experiments. We investigated the effect of this condition dependence on the ChIP-chip data by comparing genome-wide binding locations of TFBD during three different phases of growth ($OD_{600} = 0.3, 0.8$, and 1.4). Surprisingly, even though TFBD was strongly overexpressed in all three ODs, only the ChIP-chip data obtained at $OD_{600} = 0.8$ (mid phase) showed highly enriched over-representation of TFBSs within intergenic regions ($P \sim 10^{-19}$ for $OD_{600} = 0.8$, versus $P \sim 10^{-3}$ for $OD_{600} = 0.3$ and $P \sim 10^{-2}$ for $OD_{600} = 1.4$), a stringent criterion which we have used throughout this and other studies (e.g. Reiss *et al*, 2008) to enrich ChIP-chip TFBS selections for likely functional binding. Moreover, the locations of the strongest TFBSs were more in agreement (within 50 nt) between the early- and mid-phase ($OD_{600} = 0.3$ and 0.8 , respectively) data ($P \sim 10^{-3}$) and between the mid- and late-phase ($OD_{600} = 0.8$ and 1.4 , respectively) ($P \sim 10^{-3}$) than between the early- and late-phase ($OD_{600} = 0.3$ and 1.4 , respectively) data ($P \sim 0.35$).

Validation of regulated transcriptional initiation from promoters in coding sequences

The proximity of chromosomal loci with multiple TFBSs and experimentally mapped TSSs within coding regions strongly indicates that the observed conditional modulation of operon organization is achieved through the activation of promoters in coding sequences. However, it is imperative to rule out alternate hypotheses, such as conditional transcript cleavage followed by differential degradation of the mRNA fragments as such a process could also result in truncated transcript(s) with a 5' or a 3' boundary within a coding sequence. Verifying our precision of mapping a transcript boundary and TFBSs with other technologies (EMSA, northern blot, 5' RACE and so on) cannot definitively refute this alternate hypothesis. Instead, the ultimate proof for functional promoters within a coding sequence is the *in vivo* demonstration of regulated transcrip-

tion initiation at these loci. To provide such evidence we developed a fluorescence-based assay using a fast-degrading variant of GFP (Reuter and Maupin-Furlow, 2004) and showed that the synthesis and degradation of this reporter accurately reflects dynamics observed at the level of transcription (Supplementary Figure 4). Next, we constructed GFP transcriptional fusions to assess the activity of promoters inside coding sequences of two genes encoding a tRNA endonuclease (VNG2210G) and a siroheme biosynthesis enzyme (VNG1775C) (Figure 6A; Materials and methods). The 100-bp long internal promoter regions were selected to include the TSS and the adjacent experimentally determined TFBS locus. Using flow cytometry, we then monitored the change in fluorescence along the growth curve (Figure 6B). Fluorescence of *H. salinarum* NRC-1 transformants with internal promoter-GFP (P_{2210G}^{int} -GFP and P_{1775C}^{int} -GFP) fusions and a no-promoter construct (NULL-GFP) was measured along the growth curve using flow cytometry and calibrated against a fluorescent bead internal standard (Figure 6C; Materials and methods). This validated functional transcription initiation from these promoters. Moreover, it also showed that the activity of the promoter was conditional on the growth phase (Figure 6E). Although it is difficult to assess every instance of internal promoters using this approach, it nonetheless validates that integrated analysis of globally determined TSSs and TFBSs coupled to the analysis of gene-expression changes can lead to the discovery of conditionally activated promoters—even when they are located inside genes and operons.

Discussion

Our analysis of genome-wide protein-DNA binding sites suggested that $\sim 10\%$ of the multi-TFBS loci fell within coding regions. To show that these TFBS have significant functional consequences on transcriptional regulation and cellular physiology, we carried out a series of systematic experimental validations. First, we analyzed the transcriptome structure of *H. salinarum* NRC-1 under dynamic conditions. By correlating locations of the transcriptional units to predicted coding sequences in the genome, we were able to discover and characterize new features within the transcriptome. Next, by integrating TFBS locations with the transcriptome structure, we were able to show that some of these internal binding sites were indeed functional in the conditional modulation of operon organization, including promoters in coding regions. Finally, using transcriptional fusions to GFP reporters we provided *in vivo* validation of growth-phase-regulated transcription initiation from two of these promoters localized in coding sequences. We will discuss in detail each of these findings and their impact on our understanding and modeling of GRNs.

New features in *H. salinarum* transcriptome: increasing and detailing the parts lists

By analyzing the transcriptome of *H. salinarum* NRC-1 using high resolution and under dynamically changing growth conditions, we were able to assign TSSs to 64% of all annotated genes, TSSs to 46% of the genes and verify the

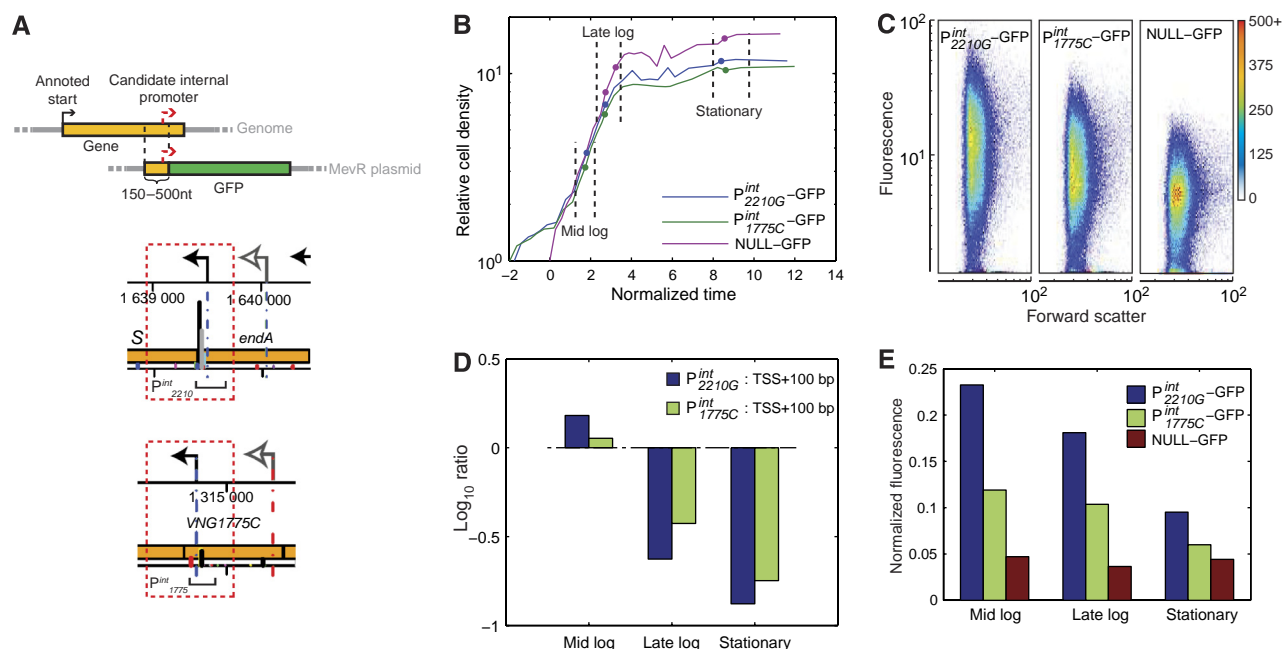


Figure 6 GFP expression validates promoters localized in the coding regions. (A) Internal promoter–GFP fusion construction strategy. The 150–500 nt regions upstream of internal transcription start sites (within tiling probe error) were fused to a GFP coding sequence on a mevinolin resistance (MevR) selectable expression plasmid. Selected internal promoters located within coding sequences of *VNG2210G* and *VNG1775C* used for GFP-expression validation are shown. (B) Sampling points along transformants growth curves. Batch cultures of strains carrying these internal promoter–GFP transcriptional fusions were sampled at mid-log, late-log, and stationary phase. For purpose of comparison, the growth curves were normalized in time relative to the characteristic growth rate observed during exponential growth. (C) The 2D densitometric scattergram of fluorescence versus forward scatter for cells with active internal promoters. Population density increases from blue to red. (D) Growth-phase-dependent transcriptional activity of the internal promoters was measured as the mean signal intensity for probes covering a region of 100 nt downstream of the internal TSS. (E) GFP production was used as proxy to validate that the growth-phase-dependent change in transcription observed in panel D was due to regulated transcription initiation by the internal promoters. The plots indicate normalized mean population fluorescence values during various growth phases (calculated from distributions shown in panel C for samples noted in panel B).

expression of 203 operons. Notably, the average distance of ~24 nt between these TSSs and experimentally mapped locations of TFBSs for one or more of 11 TFs is consistent with earlier knowledge of the relative position of the pre-initiation complex from the TSS. One important outcome of mapping TSSs and TTSs was the discovery of 5' and 3' UTRs for genes and operons. The observed absence of a 5' UTR in most of the genes with an identified TSS suggests either internal SD translation signals or more likely, an alternate (eukaryotic-like) mechanism of translation initiation. In contrast, most transcripts with an experimentally detected TTS had a 3' UTR, which was on average longer than those determined by Brenneis *et al* (2007). This difference can be explained by the smooth decay observed in the signal at the 3' end of the transcripts (Figures 1A and 2), which suggests that for a given gene, there are mRNA populations with the same 5' end but different 3'-end locations. The shorter messages are usually more abundant and have higher probability of being selected for 3'-end determination by sequencing approaches (Brenneis *et al*, 2007). We hypothesize that this smooth signal decay at the 3' termini of most transcripts is a product of imprecise termination, degradation, and/or paused or incomplete elongation complexes. Overall, ~5% of the *H. salinarum* *NRC-1* genome seems to be transcribed simultaneously in both strands. We also observed an overlap of 3' ends of 137 transcript pairs, a phenomenon that has also been observed in yeast at a similar scale (Nagalakshmi *et al*, 2008), but the functional implication of this overlap remains to be investigated.

By correlating the transcriptional units contained within pairs of TSS and TTS with chromosomal coordinates of predicted genes (Ng *et al*, 2000) and experimentally mapped peptides from large-scale proteomics studies (Van *et al*, 2008), we were able to revise the translation start site for 61 genes and detect 10 new protein-coding genes (Supplementary Table 4). This highlights the importance of constant genome re-annotation on the basis of evidence presented by new high-throughput experimental data. Another important feature was the identification of 61 new putative ncRNAs in *H. salinarum* genome. Although the physiological roles and mechanism of action of specific ncRNAs remain to be uncovered, the significant correlation (positive or negative) between the profiles of the ncRNAs and the antisense strand (Figure 3) are consistent with the characterized roles of ncRNAs in the regulation of their cognate antisense transcripts. The discovery of these ncRNAs represents new information in the parts lists of regulatory elements encoded by the *H. salinarum* *NRC-1* genome.

Mapping of all these new features of the *H. salinarum* *NRC-1* transcriptome is expected to pave the way toward a detailed functional and mechanistic analysis of GRNs, thereby improving global models of cellular behavior.

Dynamics of operon organization

By observing the dynamics of the transcriptome structure, we noted that the organization of some operons seemed to be

conditionally modulated. To assess the global prevalence of such operons, we devised a quantitative measure for classifying any operon as conditional, by integrating both the data from the transcriptome structure ('tiling score') with correlations derived from expression profiles of *H. salinarum* *NRC-1* genes in 719 microarray experiments (see Materials and methods). Using these measures, we classified 43% of the measured operons as condition dependent (Figure 4F, red dots; Supplementary Table 6). There was a strong functional link between transcription factor binding inside operons and their classification as 'conditional' ($P < 10^{-9}$).

The conditional modulation of the operon *arcBCD* (Figure 4A) involved in arginine fermentation could be validated independently. Northern-blot experiments from (Ruepp and Soppa, 1996) showed the existence of the three possible transcripts suggested by our analysis. The failure to predict an alternate promoter for *arcB* from co-expression and genome organization analysis (Price *et al*, 2005) (Figure 4A (c)) emphasizes the importance of an integrated approach that incorporates TSS and protein-DNA interactions to identify detailed mechanistic information for modeling GRNs. We have provided further evidence that two newly discovered internal promoters inside the coding sequences of *VNG2210G* and *VNG1775C* (Figure 6) can also drive regulated transcriptional initiation during complex changes associated with growth.

It is arguable whether, in some cases, our data simply refute the initial prediction of operon organization (Price *et al*, 2005). However, operons with low correlation and high tiling score have high probability of being conditionally co-transcribed, as there is no difference in their absolute transcript levels, suggesting that these genes are transcribed as a polycistron in some, if not in most, conditions. Many operons with low overall correlation and low tiling score still present meaningful co-expression of genes in specific conditions (see Supplementary information 1 at http://baliga.systemsbio.org.net/regulatory_logic/). Likewise, many operons have high correlation but low tiling score, suggesting that they have identical relative transcript levels (and are probably co-regulated) even if their absolute transcript levels differ. On the basis of this evidence, we posit that considering operons as dynamic entities is more appropriate than refuting the initial prediction, given that the currently available data sets do not exhaust the universe of possible environmental perturbations. This raises interesting questions on how the annotation databases will evolve to represent the complicated dynamics of biological features.

Physiological implications for conditional modulation of operon structures

Modulation of transcript levels within certain operons suggests a change in stoichiometry or composition of subunits within protein complexes. Alternatively, it can also be a mechanism for maintaining stoichiometry of a complex with differential turnover or translation rates of specific subunits (Hayter *et al*, 2005). We illustrate this with two examples:

1. In the *sdhCDBA* operon (Figure 4C), which encodes four subunits of succinate-dehydrogenase (SDH) enzyme complex, the Fe-S, cytochrome *b*, and membrane anchor

subunits (SdhC, D, and B, respectively) are downregulated at high cell densities, whereas the expression of the FAD-binding flavoprotein component (SdhA) remain unchanged (Figure 4C-c). Downregulation of SDH at later phases of growth correlates with a drop in oxidative phosphorylation and concomitant reduction in cellular ATP levels (Schmid *et al*, 2007). Interestingly, differential regulation of the SdhA subunit relative to the three other subunits has also been observed in mitochondria and has been linked to O₂ sensing (Piantadosi and Suliman, 2008). Although the implications of this differential regulation are bound to be different in *H. salinarum* *NRC-1*, it nonetheless suggests potential physiological consequences of changing stoichiometry of SDH subunits.

2. In the *dppFDB2C2* operon for a dipeptide ABC transporter (Figure 4D), all subunits, including the periplasmic and permease components (DppD, B2, and C2) with the exception of the ATPase subunit (DppF) were upregulated at higher cell densities (Figure 4D(c)). Among other speculations, including possible subunit exchange with other ABC transport systems, it is possible that this complex may function without an ATPase subunit (Hebbeln *et al*, 2007) to conserve a limited ATP pool at higher cell densities (Schmid *et al*, 2007) by taking advantage of a nutrient rich environment.

The association of a TFBS with a TSS internal to an operon strongly suggests that the operon's genes are conditionally transcribed through alternate promoters. Alternative mechanisms that could result in such behavior include post-transcriptional cleavage followed by differential turnover of mRNAs, intra-cistronic transcription termination (Adhya, 2003; Lee *et al*, 2008), or even the condensation state of the genome by chromatin proteins in regulating gene expression (Reeve and Sandman, 2007). Although specific consequences of this phenomenon on the function of SDH and the ABC transporter will require further investigation, these observations nevertheless challenge our assumption that the operon organization of genes encoding these complexes reflects static subunit composition and fixed stoichiometry in diverse environments. Our findings reinforce the fact that even simple prokaryotes possess complex mechanisms to fine tune the expression of genes in an operon through prevalent use of alternate promoters and/or terminators that are combinatorially induced or repressed in response to dynamically changing environments (Adhya, 2003).

The significance of conditional activity of transcriptional promoters

Although ~57% of the operons were classified as not condition dependent by our analysis (Figure 4F, black dots), we cannot rule out their conditional modulation in environments yet to be investigated. This is because transcription is a regulated and dynamic process with activities of any given TF dependent not only on its own abundance in the cell but also on the simultaneous presence and availability of relevant cofactors, accessibility of binding sites and/or absence or diminished activity of other competing factors. Intragenic binding sites could also indicate re-association of TFs with the

transcription complex, aiding the polymerase in the recognition of pausing or termination signals (Reppas *et al*, 2006; Lee *et al*, 2008) or may even indicate previously unknown activity of GTFs (Wade *et al*, 2007). Our ChIP–chip experimental data rely on the overexpression of a given TF from a heterologous promoter on a plasmid, which might alter the relative concentrations of the TFs in the cell. In order to overcome this experimental constraint, we took a highly conservative approach, where only multi-TFBS loci were considered for further analysis.

Notably, there were numerous examples where *bona fide* associations of TFBSs and TSSs or TTSs were not made by our conservative automated procedure (see Materials and methods). For instance, although some TFBSs presented weak binding-signal intensity, they could be manually associated with TSSs or TTSs, suggesting they too might be functional; and *vice versa*, weak TSSs or TTSs could be assigned to nearby TFBSs. Furthermore, by charting growth-phase-dependent changes in genome-wide binding patterns of TFBd we also provided further evidence for the conditional nature of TF binding. Although this shows some limitations in the coverage afforded by our automated approach and the single data set covering only growth-related transcriptional changes, it also gives a perspective on how to incorporate such valuable information with less stringent modeling approaches that integrate orthogonal evidences to mechanistically define GRNs.

In other words, protein–DNA binding is probabilistic and combinatorial, depending on the relative abundance of TFBSs, TBP, and TRs under specific environmental conditions, thus, when we observe a TFBS that is weak or not associated with a transcript boundary or a strong promoter consensus (Muller *et al*, 2007), it does not necessarily mean that it is not functional. In fact, some weak-affinity TFBSs in yeast have been shown to be functional (Tanay, 2006). Only given sufficient experiments for localizing most TFBSs in a wide array of environments, one could map these types of conditional interactions to construct a comprehensive map of dynamic transcriptional regulatory mechanisms at all promoters.

Conclusions

Historically, studies of transcriptional regulation have often focused on TFBSs upstream to coding sequences. Furthermore, TFBSs inside coding regions are usually viewed as spurious or non-functional and used as a quality control metric for genome-wide ChIP–chip experiments. Computational (Ward and Bussemaker, 2008) and experimental (Lee *et al*, 2002) analyses of promoters restricted to intergenic regions add a significant bias in the distribution of documented TFBSs in the genome, reinforcing the overall assumption that transcriptional regulation occurs exclusively in intergenic regions.

Interestingly, many studies that focus on a single gene or operon do find internal promoters (Tsui *et al*, 1994; Guillot and Moran, 2007). We have significantly extended these findings, by showing that the widespread and conditional affinity for several TFs does not discriminate between coding and intergenic regions. It also shows how a simple prokaryote can use the same set of genes in different combinations to elicit

different responses according to the environmental challenge. Given recent reports of extensive TF binding within coding regions in many organisms (Tanay, 2006; Zhu *et al*, 2006; Yochum *et al*, 2007; Shimada *et al*, 2008), evidence is mounting to suggest that this is a general phenomenon. This is not surprising given that the genome is known to encode multiple levels of information within the same sequence (Itzkovitz and Alon, 2007)—here TF binding and gene coding.

Irrespective of the specific underlying mechanism(s), our observations of widespread modulation of operon architecture, as well as transcription initiation and termination inside genes, etc. all constitute evidence that archaea can intersperse regulatory logic within their coding sequence and blur the boundaries between coding and non-coding elements. We have shown that it is possible to use new high-throughput technologies to find these biologically important instances in which transcriptional regulation does occur within coding sequences and, furthermore, that it is possible to globally characterize specific regulatory mechanisms responsible for these phenomena. Combined with new high-throughput sequencing technologies, our results will expand the view of genetic information processing that can be investigated at high resolution (Nagalakshmi *et al*, 2008; Wilhelm *et al*, 2008). These data will enable construction of mechanistically accurate models for reliable systems re-engineering of biological circuits.

Materials and methods

Strains, culturing, and growth conditions

H. salinarum NRC-1 growth-curve experiments were conducted in CM media, in a water bath incubator at 37°C with agitation of 125 r.p.m. Reference samples were cultured under standard growth conditions (Baliga and DasSarma, 1999), at mid-log phase ($OD_{600} \approx 0.6$), as well as all the strains used for ChIP–chip experiments (Facciotti *et al*, 2007).

High-resolution tiling array construction, RNA hybridization, and reference normalization

Whole-genome high-resolution tiling arrays for *H. salinarum* NRC-1 were designed with e-Array (Agilent Technologies), using strand-specific 60mer probes tiled every 20 nt for the main chromosome (NC_002607) and every 21 nt for the plasmids pNRC200 (NC_002608) and pNRC100 (NC_001869), consisting a total of 244K probes, including manufacturers' controls. The microarrays were printed by Agilent technologies and hybridized to total RNA, which was isolated using *mirVana* miRNA Isolation kit (Ambion) and direct labeled with Alexa547 and Alexa647 dyes (Kreatech) (Baliga *et al*, 2004). We used direct chemical labeling of RNA (Baliga *et al*, 2004) to avoid enzymatic labeling artifacts (Perocchi *et al*, 2007) and enable strand-specific signals for transcribed segments. Hybridization and washing were carried out according to array manufacturer's instructions. Arrays were scanned in ScanArray (Perkin Elmer) and spot finding was carried out using Feature Extraction (Agilent Technologies). Two biological replicates were sampled and dye-flip experiments were conducted for each sample. Resulting intensities were quantile normalized across all experiments. Log ratios were calculated for each probe (growth-curve sample/reference). The reference-RNA signals were quantile normalized and then jointly normalized by sequence content using a linear model similar to that of Johnson *et al* (2006). This model attempts to capture the effect on hybridization signal or efficiency from duplicate probes (cross-hybridization), G-C content, and sequence-specific factors. The 'sequence-based' correction was subtracted from the probe intensities and resulted in a

reduction by ~10% in residual sum-of-squares between the intensities of neighboring probes. Interactive visualization of the data was carried out in the Gaggle Genome Browser (Bare *et al.*, in preparation), available at http://baliga.systemsbiology.net/regulatory_logic/.

ChIP–chip experiments and analysis

ChIP–chip experiments were carried out for all TFBs (TFBa, TFBb, TFBc, TFBd, TFBf, and TFBg), one TBP (TBPb) and three TRs (Trh3, Trh4, and VNG1451C) in *H. salinarum* NRC-1 using the HaloSpan array (Facciotti *et al.*, 2007), which consists of 500 nt PCR products of successive regions of *H. salinarum* NRC-1 genome. The data for all TFBs were retrieved from Facciotti *et al.* (2007). For TFBa, TFBd, and TFBf, data for additional biological replicates were also acquired using 13-nt resolution tiling arrays on the Nimblegen platform (for TFBd, two such distinct biological replicates were acquired). The TRs encoding genes were cloned in pMTFCmyc vector; chromatin immunoprecipitation and identification were carried out as described by Facciotti *et al.* (2007). Binding locations were defined by applying the *MeDiChI* algorithm (Reiss *et al.*, 2008) to each data set. This regression-based method deconvolves the ChIP–chip enrichment ratios along the genome by fitting them with a ‘peak profile’ model of binding events, assuming a distribution in enriched DNA fragment lengths. It was shown that *MeDiChI* can increase the effective resolution of TFBs locations by a factor of five relative to the probe spacing of the tiling array, even for overlapping peaks (Reiss *et al.*, 2008). *P*-values reported by *MeDiChI* (based upon peaks detected in bootstrap-resampled data that statistically seem to contain only noise; see (Reiss *et al.*, 2008)) for each data set were converted to LFDR estimates through a semi-parametric two-component mixture model (Robin *et al.*, 2007). A comparison of the peak intensities derived from *MeDiChI* for all three TFs (TFBd, TFBf, and TFBa) for which there were biological replicate measurements using both microarray platforms (500 nt resolution spotted arrays versus 13 nt resolution Nimblegen arrays) (Supplementary Figure 5) provided strong validation (with R^2 of 0.66, 0.52, and 0.81, respectively) of most (~500) TFBs included in the analysis, and even for TFBs with LFDR > 0.1. The 318 multi-TFBs loci described in Results and Discussion were computed by locating peaks in a kernel density estimate (bandwidth=50 nt) of all TFBs with LFDR < 0.1 across the genome. Only density peaks generated by > 2 individual TFBs were counted. Monte Carlo simulations were used to estimate the expected fraction of TFBs, which would be detected in intergenic regions, as a function of detection positional uncertainty σ , and FDR f , given that the true TFBs locations fall only in intergenic regions across the genome. In these simulations, n TFBs were simulated by placing $n(1-f)$ TFBs in intergenic regions and nf in annotated coding regions, and Gaussian-distributed random offsets ($\pm \sigma$ in nucleotides) were added to each simulated TFBs location. We used $n=20\,000$ for our simulations. A binding event was considered internal to a transcribed or annotated coding region only if it was internally localized at a distance of > 50 nt from the respective region’s boundaries.

The microarray data reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (GEO accession no. GSE13150).

Identification of probes hybridizing with transcribed regions of the genome

Probes in the tiling arrays were assessed as to whether they were complementary to a region that is transcribed in one or more of the observed conditions. We integrated the following probe measurements: (a) their log intensities in the 54 reference-RNA tiling arrays, (b) their relative changes across the growth curve in the 12 growth-curve tiling arrays, and (c) their Pearson correlations with the changes of their two neighboring probes across the growth curve (McGrath *et al.*, 2007) into an iteratively reweighted logistic regression model that used annotated coding regions as the ‘training’ set. The resulting model was used to estimate a probability that each probe was complementary to a transcribed region.

Integrated, multivariate segmentation defines transcript boundaries

Regression trees (CART; Breiman *et al.* (1984)) were used to partition the tiling array data (log probe intensity values) into regions of constant intensity, separated by abrupt ‘break points’, by fitting a constant value to a large, contiguous region, and recursively dividing the regions to significantly improve the residual sum-of-squares. The number of splits (and hence the complexity of the model) was determined using 100-fold cross-validation, to choose the most parsimonious model within 1σ from the optimal one. The relative likelihood of each break was estimated using 100 bootstraps with symmetric wild resampling (as in Reiss *et al.* (2008)). Each segment was constrained to contain no fewer than five probes, restricting the procedure to detect only larger (≥ 100 nt) segments (putative transcripts). Using the multivariate implementation of this procedure in the *mvpart* R library, we could apply the procedure simultaneously to all tiling arrays described above, enabling us to constrain the segments in an integrated manner using the reference-RNA and growth-curve tiling arrays, as well as the growth-curve correlations and the probe transcription probabilities. The maximum resolution of the derived transcription break points is no better than the tiling array resolution (here, 20 nt). The resulting breaks were subsequently classified into transcription ‘starts’ and ‘stops’ based upon whether the signal increased or decreased across the break in both the RNA references and the probe transcription probabilities.

Detection of putative non-coding RNAs

The procedure described above for segmenting the data and identifying transcriptional start/stop sites was constrained to omit smaller (≤ 100 nt) transcripts. To identify putative non-coding RNAs (ncRNAs), including smaller ncRNA candidates, we individually partitioned each growth-curve sample data set (log ratios of the growth-curve samples relative to the reference RNA) using recursive partitioning trees as previously described. For each sample, *P*-values for each segment were computed relative to the log ratios of reference-RNA samples localized in the segment’s coordinates. A segment was classified as a putative non-coding RNA if it presented a high probability of being expressed (*P*-value < 0.05), its neighboring segments were not differentially expressed (*P*-value > 0.05, in order to filter out possible UTR regions of genes), and no annotated gene or repeat overlapped the segment’s coordinates. Further filtering was carried out through the model used to estimate a probability that each probe was complementary to a transcribed region (see section ‘Identification of probes hybridizing with transcribed regions of the genome’), and segments with *P* > 0.05 were discarded. Many ncRNAs were complementary to repeat regions of the genome, so all duplicate ncRNA candidates were removed if they contained a 12-nt contiguous sequence similarity with any other ncRNA candidate.

Peptide atlas update

H. salinarum NRC-1 Peptide Atlas was updated with the addition of three new experiments corresponding to cultures grown under standard conditions. Sample preparation and mass spectrometry analyses were carried out as described by Van *et al.* (2008), resulting in an additional 30 mass spectrometry runs and 33 986 tandem mass spectra. A new search database was constructed, including sequences from newly identified transcribed regions in the tiling array experiments. The updated version of *H. salinarum* NRC-1 peptide atlas is available at https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/buildDetails?atlas_build_id=130, including a total of 527 mass spectrometry runs and 121 618 tandem mass spectra.

Identification of conditional operons

For each of the predicted operons obtained from Price *et al.* (2005), three different statistics were computed in a pairwise manner over all genes in that operon: (1) the $\log_{10}(P\text{-value})$ for the two-sample Student’s *t*-test of the mean levels of the probes complementary to each

pair of genes; (2) the Spearman rank correlation of the gene-expression profiles across 719 microarray experiments covering several diverse environmental perturbations (oxygen (Schmid *et al*, 2007), transition metals—Mn, Fe, Co, Ni, Cu, and Zn (Kaur *et al*, 2006), UV (Baliga *et al*, 2004) and gamma (Whitehead *et al*, 2006) radiation, interaction with *Dunaliella salina*, growth curve in CM media and in defined media, light–dark cycle, and oxidative stress—H₂O₂ and paraquat (unpublished; see <http://gaggle.systemsbiology.net/projects/halo/2007-04>), and (3) the Spearman rank correlation of the genes' tiling array probes over the growth curve. Thus, conditional operons (as opposed to classical operons) were identified on the basis of (1) the similarity in expression levels of the probes for each gene in the operon in the tiling array data, and (2) co-expression of the operon's genes across 719 microarrays. To obtain a probability that each operon is conditional, we computed the minimum values of each of these statistics for each operon (resulting in three 'scores' per operon), and applied a quadratic discriminant classifier to these scores, using a set of 73 manually identified conditional operons as the training set. A probability cutoff of $P=0.64$ was chosen to minimize the false classification rate for the manually classified training set. This protocol resulted in a total of 123 classified conditional operons. Finally, the cumulative hypergeometric distribution was used to assess the P -value for the over-representation of TFBSs internal to the 123 conditional operons (from the ChIP–chip data for all GTRs and TFs described above) versus the number of TFBSs internal to the 176 non-conditional (classical) operons.

Construction of promoter–GFP fusions and evaluation of promoter activity

A 150–500bp region surrounding the TSS localized in coding sequences of VNG2210G and VNG1775C was PCR amplified (primers VNG2210G-F: 5'-CGAAAACCGGATTCAAGTTC-3', VNG2210G-R: 5'-ATCGTGTCTGTGTCTGTC-3', resulting in a 208-bp PCR product corresponding to region 1 639 638–1 639 431 in *H. salinarum* main chromosome and VNG1775C-F: 5'-CTTCGGTCGACAGGGTTATC-3' and VNG1775C-R: 5'-TGTCGACCAATCTACGTCGC-3', resulting in a 162-bp PCR product corresponding to region 1 314 877–1 314 716 in *H. salinarum* main chromosome) and fused to a GFP coding sequence on a MevR selectable expression plasmid. *H. salinarum* NRC-1 cells were transformed and selected on CM agar containing 20 µg/ml mevinolin. Cells were sampled at mid-log, late-log, and stationary phases. On sampling, the cells were simultaneously washed, diluted to a nominal density of OD₆₀₀ 0.2, and fixed in a basal salt solution with 0.25% (w/v) formaldehyde. Cells were incubated in the fixative for 10 min at 4°C, followed by a second wash in basal salt solution. The fixative concentration was previously determined to adequately arrest cell function while also preserving GFP fluorescence and the combined wash steps served to remove as much of the peptone-based growth medium as possible to reduce fluorescence background. The dynamic range of the flow cytometer (InFlux, Cytopia/BD) was calibrated using fixed, non-fluorescent *H. salinarum* NRC-1 cells and 1-µm Y/G fluorescent beads (Polysciences, Inc). Before running on the flow cytometer, 1-µm beads were spiked into each sample to a final density of 1×10^7 beads/ml (approximately ten-fold less than the nominal cell density) to serve as an internal calibration standard. The mean fluorescence from 100 000 cells in each sample was normalized relative to the bead fluorescence level.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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